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Neuronal pentraxin 2, brain atrophy and cognitive decline

Ashley Swanson
Iowa State University

Neuronal pentraxin 2, brain atrophy and cognitive decline

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Ashley Swanson

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Auriel Willette, Major Professor
Matthew Rowling
Marian Kohut

The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Chronic neuroinflammation is thought to potentiate medial temporal lobe (MTL) atrophy and memory decline in Alzheimer's disease (AD). It has become increasingly important to find novel immunological biomarkers of neuroinflammation or other processes that can track AD development and progression. Our study explored which pro- or anti-inflammatory cerebrospinal fluid (CSF) biomarkers best predicted AD neuropathology over 24 months. Using Alzheimer's Disease Neuroimaging Initiative data (N=285), CSF inflammatory biomarkers from mass spectrometry and multiplex panels were screened using stepwise regression, followed up with 50%/50% model retests for validation. Neuronal Pentraxin 2 (NPTX2) and Chitinase-3-like-protein-1 (C3LP1), biomarkers of glutamatergic synaptic plasticity and microglial activation respectively, were the only consistently significant biomarkers selected. Once these biomarkers were selected, linear mixed models were used to analyze their baseline and longitudinal associations with bilateral MTL volume, memory decline, global cognition, and established AD biomarkers including CSF amyloid and tau. Higher baseline NPTX2 levels corresponded to less MTL atrophy [R^2= .287, p<.001] and substantially less memory decline [R^2=.560, p<.001] by month 24. Conversely, higher C3LP1 modestly predicted more MTL atrophy [R^2=.083, p<.001], yet did not significantly track memory decline over time. In conclusion, NPTX2 is a novel pro-inflammatory cytokine that predicts AD-related outcomes better than any immunological biomarker to date, substantially accounting for brain atrophy and especially memory decline. C3LP1 as the microglial biomarker, by contrast, performed modestly and did not predict longitudinal memory decline. This research may advance the current understanding of AD etiopathogenesis, while

expanding early diagnostic techniques through the use of novel pro-inflammatory biomarkers, such as NPTX2. Future studies should also see if NPTX2 causally affects MTL morphometry and memory performance.

Keywords: Alzheimer's disease; medial temporal lobe; inflammation; immunology; amyloid; tau; memory; biomarkers; NPTX2; C3LP1

CHAPTER 1: INTRODUCTION

Thesis Organization

My thesis begins with a review of the literature on Alzheimer's disease, neuroinflammation, synaptic plasticity, as well as pro-inflammatory cytokines and pentraxins, with their speculated and probable immunological role in the etiopathogenesis of Alzheimer's disease being included.

The next section of my thesis consists of the methods conducted during my study, followed by the results obtained and a discussion of the meaning of these results. Lastly, I've included a conclusion of the study to summarize the implications for this novel study for the future of the field of AD biomarker and proteomics research.

Purpose of Study

The vast majority of research to date has been on studying the upregulation of pro-inflammatory cytokines and their specific role in the chronic neuroinflammatory mechanisms that underlie AD pathogenesis. My study proposes to highlight the need for understanding that these cytokines are functionally pleiotropic in their regulation of neuroinflammation and cannot simply be thought of as "good" or "bad" protein phenotypes.

Therefore, it is worthwhile to explore established and novel CSF pro- or anti-inflammatory biomarkers that are associated with baseline and longitudinal AD neuropathology and memory performance in aged participants across the AD spectrum. In this study, we used data from a database called Alzheimer's Disease Neuroimaging Initiative (ADNI) to analyze 285 aged adult subjects at baseline and through months 6,

12, and 24. CSF peptide biomarkers of neuroinflammation were isolated from baseline liquid chromatography/multiple reaction monitoring mass spectrometry (LC/MRM-MS) (http://adni.loni.usc.edu/about/centers-cores/biomarker/), as well as a CSF multiplex protein array.

Based on the initial stepwise regression analyses conducted (see Section 2.8 in Methods and 3.2 in Results), NPTX2 and C3LP1 were selected for significance and further examined. The selected biomarkers were analyzed to explore their baseline and longitudinal associations with gray matter (GM) volume in bilateral MTL and a memory factor, as well as global cognition and established AD biomarkers such as CSF Aβ1-42 and tau, and phosphorylated tau (ptau).

CHAPTER 2: LITERATURE REVIEW

Alzheimer's disease

Alzheimer's disease (AD) is clinically characterized by a number of components including global cognitive impairment, memory decline, loss of activities of daily living, a neural accumulation of tau and amyloid, and neuronal death, which manifest as profound atrophy of important brain regions. In order for one to be clinically diagnosed with AD, one needs evidence of memory loss and a deficit in at least one other cognitive domain, such as loss of executive function[1].

Historically, AD brain pathology has been characterized by an accumulation of two primary protein aggregate suspects gone awry: extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles (NFT) [2]. This led to the amyloid cascade hypothesis, whereby amyloid precursor protein (APP) undergoes cleavage by proteolysis resulting in beta-amyloid fragments that clump together and form plaques, with increased concentrations found in the AD brain [2,3]. More recently, the hypothesis has evolved to state that perhaps the Aβ that is not isolated in plaques actually drives the disease [4] and thus also plays a role in hippocampal function and long-term potentiation (LTP)[5].

Tau is a microtubule-associated protein (MAP), and through various mechanisms becomes hyperphosphorlyated in AD and in the vast majority of research is thought to contribute to neurodegeneration by disrupting normal tau proteins [6]. However, recent AD research aims to debate the extent of how bad tau hyperphosphorylation really is, with it providing probable cause that some tau phosphorylation may be neuroprotective and actually combat against Aβ-induced excitotoxicity[7]. Research has proposed that Aβ plaques have the ability to trigger the formation of toxic tau tangles, which results in the

two aggregates working together to disrupt normal cell function and signaling pathways in the brain [8,9]. Today, while Aβ plaques and NFT are the only hallmark lesions that are being used for clinical diagnosis, with a certain number of lesions being required for diagnosis [1,3], new proteomics research has suggested a vastly different paradigm to fully understand the many factors that contribute to the etiopathogenesis and progression of AD rather than the single "amyloid and tau" pathology hypothesis[10].

There is a current rise in AD prevalence, with research projecting that by the year 2050 one new case of AD will develop every 33 seconds and nearly 1 million new people will develop it annually in the United States[11]. The total costs for caring for this AD/dementia related population in 2011 was around $186 billion per year in the United States and is slated to increase to about $1.1 trillion dollars per year by 2050[12]. The aging baby boomer generation, along with an increased life expectancy, is what will largely propel the drastic increase in AD rates. Research efforts have been focused on the biological implications in this disease, as well as potential therapies that could prevent or delay it. Currently, there are no effective preventative treatments or cures for AD, only symptomatic remedies[13]. While most of the therapies in research are aimed at targeting the classical Aβ plaques and Tau tangles, other mechanisms of pathological treatments that could decrease Aβ and Tau tangles as a secondary effect and block progression of the disease are focusing on modulation of oxidative damage, cholesterol homeostasis and inflammation[13].

Neurodegenerative Pathology

AD is typified by progressive medial temporal lobe (MTL) atrophy and memory decline[14]. Neurodegenerative processes that contribute to this occur in specific brain areas like the neocortex and limbic system and are characterized by synaptic damage and neuronal death, with these changes corresponding to the classical cognitive impairment and memory loss associated with AD[10,15]. AD neurodegeneration also results in decreased synaptic plasticity and neurogenesis, which reveals that the etiopathogenesis of AD on the brain could affect two neurophysiological factors: a degradation of mature neurons and a decreased generation of new, functional neurons[15].

The molecular process of synaptic plasticity, specifically the formation of new synaptic connections, an alteration of gene expression and increased protein synthesis, is a crucial component to the conversion of a short term memory to the storage of a long term memory in the brain, specifically in the hippocampus, located in MTL[16–18]. The modulatory physiological process of synaptic plasticity is a complex mechanism that consists of inotropic glutamate receptors, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), cohesively having an impact on the post-synaptic response to allow for acquisition of memory in the hippocampal region [19–21]. Decades ago, scientists discovered the neurophysiology of how synaptic plasticity contributes to LTP, a mechanism that occurs along the perforant path, a known neural pathway that connects the entorhinal cortex to the hippocampal region and is important in mediating the processes of both spatial memory learning and consolidation[22]. It is important to understand that effects on the storage of information encoded through synapses in the brain arise from changes in synaptic plasticity on a

specific set of neurons, rather than on one synapse[23]. The synaptic plasticity and memory hypothesis (SPM), first characterized by Martin et al [24] proposes that the concept of memory formation occurs through a reactivation of 'traces' of memory, which is based on changes in the strength of synapses, effectively known as activity-dependent synaptic plasticity [25]. Long term potentiation and long term depression (LTD), a strengthening and weakening of the efficacy of synapses respectively, occurs at synapses through an activation of neurotransmitter receptors like NMDA and post-synaptic Calcium (Ca^{2+}) concentrations, with the amount of synaptic strength proportional to the their levels[23].

The modality for the degradation of synapse function in AD is still not understood and further research is needed to understand the physiology behind the loss of synaptic transmission that underlies cognitive deficits[26]. There is a current amyloid hypothesis that proposes that synaptic toxicity, which is a result of Aβ oligomers, causes AD and leads to synaptic degradation and loss[27,28]. Both pathological cognitive disorders and age-related cognitive decline seem to be similarly related to the levels of synaptic plasticity that decrease over time, with the same neurobiological mechanism that occurs in AD paralleling what happens in normal aging, just to a greater extent. One study by Van Guilder et al. used an animal model to test their previous hypothesis, in which a decrease in synaptic transmission will correspond with decreased cognitive decline in aged rats versus adult rats[29]. In their study, they found that in aged rats the level of dysfunctional synaptic plasticity proteins that were expressed had a direct effect on hippocampal-dependent memory and learning functions, with lower expression related to decreased cognition[29].

It has also been concluded that inhibitory regulation of synaptic genes in the biological pathways of AD, including the pathways of inflammation, oxidative stress, energy homeostasis and synapse transmission, is affected by the risk factor of age and is a crucial factor in the development and progression of AD[30–32]. Changes in synaptic strength are mostly attributed to glial cells of the immune system, also known as astrocytes, because they contain neurotransmitter receptors that can regulate synaptic plasticity and transmission through a multitude of mechanisms that function to alter neuronal physiology[33].

It has become increasingly important to find novel, immunological biomarkers that can track AD development and progression. Neuroinflammation may be a useful process to examine, as it is an early and continuous feature of AD[34] that underlies neurodegeneration and cognitive deficits[35]. The study of proteomics has the potential to reveal predictive biomarkers that underlie many of the molecular pathways associated with etiology of AD, and aid in early diagnosis and treatments as well as the diagnosis of patients progressing from mild cognitive impairment (MCI) to AD[10]. More research needs to be conducted to analyze how to prevent or treat cognitive impairment by repairing synaptic plasticity alterations that occur with age-related/AD-related cognitive decline[29].

Neuroinflammation

In 2015, the emergence of neuroinflammation as a probable cause in the pathology of AD, prompted the Alzheimer's Association Roundtable to meet and analyze its mechanistic contributions to the etiopathogensis and progression of AD. They

concluded that there is a very present need for scientific research to advance the understanding of the molecular patterns of neuroinflammation that underlie the various stages of AD, and to find novel biomarkers of inflammation and innate immunity that could be used in the therapeutic prevention and treatment of AD [36].

There is widespread evidence for the role of neuroinflammatory mechanisms to underlie the neurodegenerative processes of AD[37]. Neurotoxic inflammatory mechanisms may initiate AD pathogenesis[38]. It is thought that chronic inflammation may even precede Aβ and tau pathology in late-onset AD[39]. Neuroinflammation, on the cellular subunit level, occurs through the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6) primarily from microglia, but also astrocytes, brain endothelial cells (BECs), and neurons themselves[38,40–42]. Levels of these cytokines and downstream effectors are higher in the AD brain[43] and may mediate neural atrophy over time[41]. This activity potentiates mitochondrial degradation and cell damage via release of reactive nitrogen and oxygen species[39]. Other complex factors modulate these responses, including complement proteins, anti-inflammatory cytokines, macrophage colony-stimulating factor (M-CSF), C-reactive protein (CRP), and S100β [34].

Microglial activation is most often potentiated in AD by Aβ peptides, neurofibrillary tangles, and neuronal cell degradation[38]. Microglia, the immune cells of the brain, are also known as the macrophages of the CNS with prominent roles in homeostatic regulation of synaptic plasticity and neuronal pathways and in initiation of neuroinflammation through a release of inflammatory mediators[36]. Microglia have been implicated to exercise roles in the healthy CNS through neurogenesis and synapse

formation, further highlighting their role in synaptic plasticity modulation and neurophysiological homeostasis[44,45]. Furthermore, microglial cells have come to be known as a "double-edged sword" based on their level of activation, as they can alter their phenotype based on a healthy state or neurodegenerative state, with the latter propelling microglia to retract and instigate phagocytosis causing them to lose their homeostatic, synaptic regulation[36,45,46].

Many studies now attribute the regulation of Aβ-induced neuroinflammation to AD genetic risk factors like Apolipoprotein E (APOE), where AD patients with the APOE ε4 allele have increased brain inflammation, amyloidosis, and microglial activation[38,47,48]. This cyclic process of activating cytokines and Aβ plaques, which can subsequently increase APOE expression and amyloid deposition, is thought to be an initial inflammatory mechanism that plays a role in AD etiopathogenesis[34,49]. However, while classic pro-inflammatory cytokines such as IL-1β and IL-6 can potentiate brain atrophy, they are not necessarily ideal AD biomarkers. For example, pro-inflammatory cytokines at lower concentrations induce and maintain hippocampal LTP and neural plasticity, brain homeostasis, plaque clearance via activated microglia, and tissue repair [34,50–52], where these effects are impaired at higher concentrations[53]. Since many molecules of the immune system can demonstrate opposite functions, neuropathological characterization of neuroinflammation provides little information on its actual role in AD pathogenesis[39]. Therefore, there is no easily labeled "detrimental" phenotype based on the expression of pro-inflammatory cytokines by activated microglia in the brain[54], as they exercise context-dependent pleiotropic effects based on concentration, which can vary considerably within and across individuals[55]. Furthermore, these inflammatory

mediators are inherently context-dependent, allowing for multifunctional roles in the innate immune system[48,55]. These paradigms highlight the difficulty in selecting effective pro- or anti- inflammatory biomarkers to detect and track AD. Some studies hypothesize that a more effective intervention would be to re-balance inflammatory signals to limit AD progression[56–58] as a result of the complex immunological mechanisms that participate in the inflammatory processes resulting from an activated microglial event such as aging or brain injury[49,59]

Pro-Inflammatory Mechanisms: A Role for Pentraxins and Related Biomarkers

It is also important to consider other pro-inflammatory modulatory mechanisms that do not induce chronic neuroinflammation. For example, synaptic plasticity in MTL is in part regulated by the pentraxin superfamily, such as neuronal pentraxin 2 or NPTX2[60]. Specifically, NPTX2, also known as neuronal-activity regulated protein (NARP), facilitates excitatory synapse formation, learning, and memory by clearing extracellular debris to anchor α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) channels[60–62] . In general, pentraxins, including NPTX2, have been understudied in past scientific research with few efforts being done to understand their cellular and physiological mechanisms. An underlying theme of the pentraxin research that has been conducted has found that they play a prominent role in neuronal synaptic plasticity and LTP as a novel immediate-early gene (IEG)[61,63]. Established research has revealed that the storage of long-term memories are modulated by structural and synaptic changes through a cAMP-activation of IEG's[16]. One study found that *nptx2b*, the gene for NPTX2 in zebra fish, is able to modulate synaptic plasticity in hyporcretin/orexin

(HCRT) neurons through circadian regulatory mechanisms[64], which are affected by both circadian clock balance and sleep deprivation. The concentration of NARP, a protein in rats homologous to NPTX2, is increased in the adult cortex and hippocampus of the brain with roles in neuronal growth, synaptic physiology and the associated LTP that arises from NMDA receptor activation[63]. One study concluded that NARP, as well as other associated pentraxins like Neuronal Pentraxin 1, interact and correspond to synaptic plasticity in the brain through association with AMPA type glutamate receptors, from development through adulthood [65]. NPTX2 has the potential to predict progression of MCI to AD because this biological protein is a marker in cerebrospinal fluid (CSF) of both neuronal degradation and synaptic loss [10]. NPTX2 mRNA expression has also been found to be upregulated in neurons and glia of the substania niagra and frontal cortex in Parkinson's Disease (PD), another neurodegenerative disorder, and is thought to play roles in PD dysfunction as a result of synaptic alterations in the cerebral cortex[66].

Historically, both the pentraxins and receptor (NP1, NPTX2 and NPR) had been proposed to function similar to acute phase proteins in the acute phase of immunity by binding and clearing extracellular pathogens, synaptic debris and toxins from the neurons, further elucidating their role of protection and modulation of synaptic plasticity[67,68]. Pentraxins have the ability to recognize damaged cells and instigate apoptosis to clear away cellular debris[69]. Indeed, neuronal pentraxins exercise activity-dependent synaptic plasticity roles in both neuronal and retinal cells as a result of their ability to mark synaptic sites for degradation and cellular turnover[68]. The pentraxin family is characterized by a structural motif called a pentraxin domain[70]. New research has elucidated the pentraxins' physiological regulatory effects on the immune system,

inflammation, homeostasis and apoptosis[69,70]. Neuronal Activity-Regulated Pentraxin (NARP) has also been linked to the pentraxin known as C-reactive protein (CRP) of the acute phase response due to many similarities in pentraxin structure and its function as a calcium-dependent lectin[63].

While there is an obscure amount of research that has been done on NARP and pentraxins in general, there is a wealth of research about CRP in the field of immunology and inflammation. CRP, a proinflammatory regulatory protein and a known activator of the complement C system in the acute phase response of immunity[71], is speculated to have a protective mechanistic role as it is able to modulate and balance inflammatory reactions via activation or deactivation of the C system[72]. CRP, notably the first pattern-recognition molecule (PRM) to be discovered, is an immunological pentraxin of humoral innate immunity that can lead to an activation of adaptive immunity and tissue repair[69].

As microglial activation is also important for potentiating specific aspects of AD pathogenesis[73], related biomarkers have been investigated such as Chitinase 3-like Protein 1 (C3LP1). C3LP1, a derivative of chitin protein, is a marker of macrophage/microglial activation[74–79] . Serum and CSF C3LP1 levels are increased in preclinical and early AD[74,80], further suggesting its potential utility.

Proteomics Research

Peptidomics and multiplex techniques may reveal novel immunological biomarkers of chronic neuroinflammation or other processes that best predict MTL atrophy and memory decline. There is a current need of a disease-modifying therapy for AD[13]. Due to the multifaceted etiopathology of AD, new proteomics research is needed

to advance the understanding and diagnostic tools of this disease by looking at molecular mechanisms and the physiology underlying this disease[10]. Analysis of biochemical markers that can be used to diagnose the various stages of this disease, as well as elucidation of neurobiological changes that occur throughout AD, are vital to advancing this field[10].

CHAPTER 3: MATERIALS AND METHODS

3.1. Participants

Baseline mass spectrometry and multiplex data from ADNI were available for 86 cognitively normal (CN), 135 Mild Cognitively Impairment (MCI), and 64 AD (adni.loni.usc.edu). The following ADNI data were also available for this cohort:

1. Demographics including age, sex and education;
2. Clinical diagnosis at baseline and month 24, as well as MCI conversion;
3. MRI scans;
4. CSF Aβ1-42, total tau, and phosphorylated tau (p-tau181);
5. Apolipoprotein E (APOE) ε4 genotype;
6. Global cognitive measures and factor scores.

By month 24, MCI participants were classified as either remaining stable (MCI-S, n =82) or progressing to AD (MCI-P, n =47), with the remainder diagnosed as CN. Details of the consensus procedure by the ADNI Conversion Committee are described elsewhere[81]. We chose to focus on month 24 as an endpoint for comparison to our previous work[81], and because there is much less MRI data available after month 24.

3.2. Standard Protocol Approvals, Registrations, and Patient Consents

Written informed consent was obtained from all ADNI participants at their respective ADNI sites. Site-specific institutional review boards approved the ADNI protocol.

3.3 Clinical and Cognitive Assessments

Global cognition and assessment scores for the Mini-Mental State Examination (MMSE), clinical dementia rating-sum of boxes (CDR-sob) and AD assessment scale-cognitive subscale 11 (ADAS-cog11) were examined at baseline and at 6, 12, and 24 months. Diagnoses were made by ADNI based on criteria described in the ADNI1 procedure manual (http://adni.loni.usc.edu/). A memory factor score[82] was also examined at baseline and longitudinally. Memory decline was defined as difference scores between baseline and either 6, 12, or 24 months after. Memory factor data from baseline to months 12 and 24 was missing for 0 and 27 participants respectively.

3.4. CSF Amyloid and Tau

CSF sample collection, processing, and quality control of p-tau181, total tau, and Aβ1-42 are described in the ADNI1 protocol manual (www.adni.loni.usc.edu) and elsewhere[83]. Total tau and Aβ1-42 values were not available for 3 and 1 participants respectively.

3.5. Mass Spectrometry and Multiplex Biomarkers in CSF

Data was downloaded from the Biomarkers Consortium CSF Proteomics liquid chromatography/ multiple reaction monitoring mass spectrometry (LC/MRM-MS) dataset. As described previously[84], the ADNI Biomarkers Consortium Project investigated the extent to which selected peptides, measured with LC/MRM-MS, could discriminate among disease states. Briefly, 567 peptides representing 221 proteins were targeted in a single run (Caprion Proteome Inc., Montreal, QC, Canada). Raw intensities

were derived and extensive quality control used to derive log intensities. The ADNI Biomarker core used the natural log to transform analyte values to normalize variance in the sample. Nine neuroinflammatory biomarkers were present, represented by 21 CSF peptides. A larger CSF multiplex array, containing 27 additional pro-inflammatory biomarkers (**Supplemental Table 1**), was also utilized for comparison to LC/MRM-MS (see below).

For the LC/MRM-MS panel, the nine biomarkers of interest were: Alpha-1-antitrypsin; Complement 3; CD14; IL-18; C3LP1/YKL-40; Osteopontin; C-Reactive Protein (CRP); Neuronal Pentraxin 1 (NPTX1); and NPTX2. **Supplemental Text 1** describes all nine derived peptides and protein functions specific to inflammation. Different peptides from a single protein were selected as candidate biomarkers based on peptides that best predicted diagnostic status[84], or using stepwise regression analyses and follow up validation tests (see below). Due to the relatively small number of pro-inflammatory indices in the LC/MRM-MS peptide biomarker panel, we also explored if mass spectrometry analytes selected from that panel were again selected when simultaneously testing protein biomarkers from the larger CSF multiplex assay. Briefly, a Luminex xMAP immunoassay panel (Rules Based Medicine, Austin, TX) was used to measure 159 CSF analytes, including several pro- and anti-inflammatory proteins. As shown in **Supplemental Table 1**, 27 CSF proteins were selected based on the literature linking them to one or more inflammatory processes.

3.6. MRI and Tensor Based Morphometry

T1-weighted volumes at baseline and months 6, 12, and 24 were downloaded. Bilateral MTL gray matter (GM) volume was derived, as it shows reliable atrophy over the AD spectrum and is susceptible to neuroinflammation[85]. Baseline images were processed using FreeSurfer 4.3 as described previously (see "UCSF FreeSurfer Methods" at www.adni.loni.usc.edu). Fifty-five baseline scans were rejected for analysis based on failed QC checks. Tensor Based Morphometry (TBM) was used to gauge atrophy over time. Jacobian maps were generated between baseline and either month 6, 12, or 24 volumetric scans[86]. Degree of contraction was expressed as a percentage decrease relative to baseline, reflecting progressive brain atrophy. T1-weighted scans at months 6, 12 and 24 were missing for 13, 18 and 58 participants respectively.

3.7. APOE Genotype

The ADNI Biomarker core at the University of Pennsylvania conducted APOE ε4 genotyping. We characterized participants as being "non-APOE4" (i.e., zero APOE ε4 alleles) or "APOE4" (i.e., one to two APOE ε4 alleles).

3.8. Statistical Analyses

All statistical mixed model analyses were conducted using SPSS 23.0 software (IBM Corp., Armonk, NY). All variables had homoscedastic variance and were normally distributed or log transformed.

3.8.1. Stepwise Regression: Biomarker Selection

The nine peptide biomarkers of interest (See Section 2.5) were screened using stepwise regression which, when used correctly, is useful for variable selection and model building[87,88]. The first goal was to determine which inflammation-related LC/MRM-MS biomarkers were significant predictors of MTL volume and memory performance at 24 months. A subsequent goal was take selected mass spectrometry biomarkers and use stepwise regression while incorporating multiplex proteins, to see if MRM peptides and/or multiplex proteins were selected for model building. Covariates were entered into a given model as the first step. The nine peptide analytes representing nine candidate proteins were added in a stepwise step. In models with multiplex biomarkers, they were added in a subsequent stepwise step. The default threshold of $P<.05$ for inclusion and $P>.10$ for exclusion of variables were used. Based on these regression analyses (see Section 3.2 in Results), NPTX2 and C3LP1 were the only consistently significant biomarkers, and thus became the main predictor variables for the focus of our study. Stepwise regression iterates through each potential biomarker and removes it from the model if $P>.10$, minimizing the need for type 1 error correction.

3.8.2. Linear Mixed Models: Biomarker Testing on Outcomes

The two selected peptide biomarkers, NPTX2 and C3LP1, were subsequently analyzed with linear mixed models, to determine their baseline and longitudinal associations with GM atrophy in bilateral MTL or a memory factor. We used a single model to examine the main effects of NPTX2 and C3LP1 at baseline, or their interaction with Time longitudinally, on global cognition, memory, and bilateral MTL volume. Time

was defined as change relative to baseline at months 6, 12, and 24. Similar analyses were conducted for global cognition, Clinical Dementia Rating (CDR), and CSF amyloid and tau. Longitudinal analyses of CSF tau, p-tau181 and Aβ1-42 were not performed due to lack of longitudinal ptau-181 data in ADNI. Again, the CSF samples of NPTX2 and C3LP1 used for our statistical analyses were derived from the LC/MRM-MS (see Section 2.5).

Linear mixed models, followed by least significant differences (LSD) follow-up tests, also gauged if CSF NPTX2 or C3LP1 levels differed by baseline diagnosis (CN, MCI, or AD) or MCI conversion (MCI-S or MCI-P). All subsequent models except for cognitive outcomes included the following covariates: age at baseline, education, sex, APOE ε4 genotype, and either baseline diagnosis or MCI conversion. Mixed models also covaried the random effect of subject. Models gauging global cognition, the CDR assessment, and the memory factor did not covary baseline diagnosis or MCI conversion, because these measures are directly used to diagnose participants as CN, MCI, or AD or are direct outcomes of disease diagnosis.

Finally, on an exploratory basis, interactions were examined between both NPTX2 and C3LP1 and covariates that were statistically related to them, including APOE ε4 genotype, age, and education.

CHAPTER 4: RESULTS

4.1. Demographics and Inflammation Biomarkers

Table 1 lists demographics, APOE ε4 genotype data, and other baseline sample characteristics. Based on subsequent analyses, log-transformed CSF analyte levels of NPTX2 (TESTLNALLQR) and C3LP1 (ILGQQVPYATK) are noted.

Table 1. Demographics and Summary Indices

Abbreviations: AD, Alzheimer's disease; ADAS-cog11, AD Assessment Scale-Cognitive Subscale; APOE4, apolipoprotein ε4 allele status; C3LP1, Chitinase 3-like Protein 1; CDR-sob, Clinical Dementia-Rating Sum of Boxes; MMSE, Mini-Mental State Examination; NPTX2, Neuronal Pentraxin 2.
Note: Variables are shown as mean ± standard error or frequency count.

	CN (n=86)	MCI (n=135)	AD (n=66)	MCI-S (n=82)	MCI-P (n=47)
Age	75.70 ± 5.54	74.69 ± 7.35	74.98 ± 7.57	74.77 ± 7.37	74.64 ± 7.40
Education	15.64 ± 2.97	16.00 ± 2.96	15.11 ± 2.96	15.78 ± 3.19	16.32 ± 2.58
Sex (F,M)	42, 44	44, 91	29, 37	22, 60	20, 27
APOE4 (-/+)	65, 21	64, 71	19, 47	41, 41	20, 27
CDR-sob	0.02 ± 0.11	1.56 ± 0.88	4.34 ± 1.56	1.52 ± 0.87	1.65 ± 0.94
MMSE	29.05 ± 1.02	26.91 ± 1.74	23.52 ± 1.85	26.98 ± 1.68	26.85 ± 1.81
ADAS-cog11	6.05 ± 2.90	11.72 ± 4.33	18.88 ± 6.71	11.52 ± 4.33	12.33 ± 4.34
Memory Factor	0.98 ± 0.50	-0.15 ± 0.57	-0.91 ± 0.55	-0.10 ± 0.56	-0.26 ± 0.57
C3LP1	23.03 ± 0.03	23.13 ± 0.02	23.20 ± 0.03	23.14 ± 0.03	23.10 ± 0.03
NPTX2	10.70 ± 0.08	10.62 ± 0.06	10.31 ± 0.09	10.71 ± 0.09	10.43 ± 0.11

4.2. CSF Inflammatory Biomarker Selection (Stepwise Regression)

As a first step, stepwise regression was used to select inflammatory biomarkers that best predicted memory decline and atrophy by 24 months. As described in **Supplemental Text 1**, 9 peptides representing 9 proteins were chosen as candidate inflammatory biomarkers. All peptides were log-transformed by the ADNI Biomarker Core to achieve normality[84]. Results were similar when considering the outcomes at 12 months, or when all 21 peptide analytes were entered into the stepwise step for month 24.

For memory performance by 24 months, covariates accounted for a moderate proportion of variance [Adjusted R^2=.164, F=11.10, *P*<.001]. Stepwise selection of NPTX2 [Adjusted R^2=.202, F-change=12.91, *P*<.001] and then C3LP1 [Adjusted R^2=.215, F-change=5.17, *P*<.001] significantly improved the model. Using 10 random samples of 50% of the cohort or Lasso regression to validate model selection (**Supplemental Text 2**), NPTX2 and C3LP1 were consistently selected as the only significant predictors.

For MTL volume by 24 months, a similar pattern emerged. Covariates initially explained nearly half of the variance [Adjusted R^2=.477, F=42.27, *P*<.001]. NPTX2 [Adjusted R^2=.514, F=17.62, *P*<.001] and subsequently C3LP1 [Adjusted R^2 = .546, F-change=16.79, *P*<.001] were again selected as the only significant predictors. Using stepwise regression with random sampling or Lasso regression to validate the model (**Supplemental Text 2**), NPTX2 and C3LP1 were again selected as the only significant predictors.

Finally, as described in **Supplemental Text 3**, NPTX2 and C3LP1 were selected in the stepwise step when they were iteratively added into a model with 27 CSF proteins related to inflammation from the multiplex immunoassay (**Supplemental Table 1**). These results suggest that NPTX2 and C3LP1, respectively biomarkers of inflammation-mediated excitatory synaptic plasticity[60] and macrophage/microglia[80] activity, may be useful for tracking AD neuropathology and cognitive decline and should be investigated further.

4.3. Effects of Diagnosis and Covariates on NPTX2 and C3LP1 (Mixed Models)

Having selected NPTX2 and C3LP1, their associations with clinical diagnosis and covariates were then ascertained with linear mixed models. There was a main effect of baseline diagnosis on NPTX2 [F=4.120, *P*=.017]. **Table 1** indicates a modest step-wise decrease in log-transformed NPTX2 levels from CN to AD [*P*=.005] and MCI to AD [*P*=.034], but not CN to MCI [*P*=.242]. MCI-P had lower NPTX2 levels than MCI-S [F=4.04, *P*=.047]. A main effect of baseline diagnosis on C3LP1 was also significant [F=3.32, *P*=.037]. **Table 1** indicates a modest step-wise increase in log-transformed C3LP1 levels from CN to AD [*P*<.001], MCI to AD [*P*=.045] and CN to MCI [*P*=.002]. MCI-S and MCI-P did not differ for C3LP1 values [F=0.358, *P*=.551].

For covariates, on an exploratory basis, APOE4 carriers had higher C3LP1 [F=7.81, *P*=.006], but similar NPTX2 values [F=0.15, *P*=.696]. Older age at baseline was related to higher C3LP1 [R^2=.391, F=63.91, P<.001], but not NPTX2 [F=0.38, *P*=.539]. There was a trend for more years of education predicting higher NPTX2 [F=1.77,

P=.053], but not C3LP1 [F=1.00, *P*=.317]. Sex was not a significant predictor for NPTX2 [F=0.01, *P*=.973] or C3LP1 [F=1.32, *P*=.251].

4.4. Neuropsychological Testing: Baseline and Over 24 Months (Mixed Models)

Next, the associations of NPTX2 and C3LP1 were investigated with baseline and longitudinal indices of global cognition and function, as well as memory with linear mixed models. As shown in **Figure 1**, higher baseline NPTX2 and C3LP1 levels were, respectively, related to better and worse baseline global cognitive and assessment outcomes. Specifically, higher NPTX2 levels were correlated with higher MMSE [β±SE=1.24±0.22, F=32.85, *P*<.001], lower CDR-sob [β±SE=-0.81±0.15, F=28.22, *P*<.001] and lower ADAScog-11 [β±SE=-3.34±0.54, F=38.40, *P*<.001] (**Figure 1A,C,E**). Higher C3LP1, conversely, was associated with lower MMSE [β±SE=-1.43±0.37, F=15.26, *P*<.001], higher CDR-sob [β±SE=1.18±0.26, F=21.13, *P*<.001], and higher ADAS cog-11 scores [β±SE=4.45±0.92, F=23.55, *P*<.001] (**Figure 1B,D,F**). Similar patterns were seen across time (see **Supplemental Text 4**).

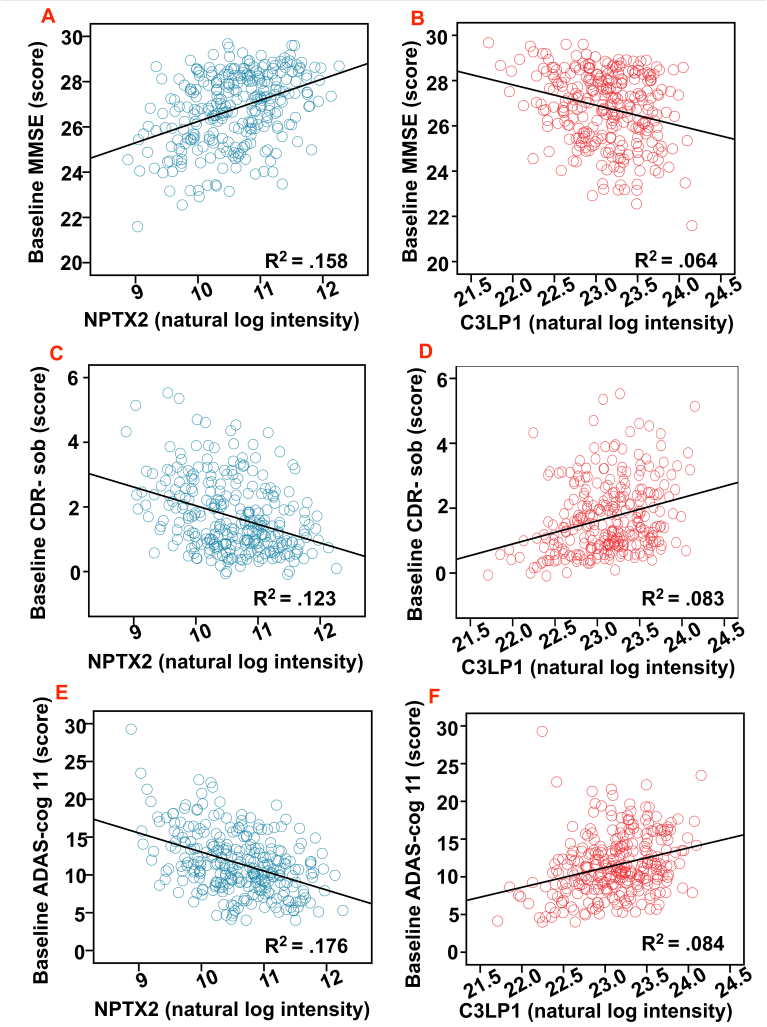


Figure 1. Mass Spectrometry Biomarkers and Baseline Global Cognition

Associations between baseline global cognitive and assessment outcomes with baseline CSF NPTX2 or C3LP1. Blue and red circles respectively correspond to NPTX2 and C3LP1 CSF values in predicting MMSE (A,B), CDR-sob (C,D), and ADAS-cog11 (E,F). The R^2 value reflects the proportion of variance in cognitive scores explained by each biomarker. Covariates included age at baseline, sex, Apolipoprotein ε4 genotype, and education. ADAS-cog11, Alzheimer's Disease Assessment Scale-cognitive subscale 11; C3LP1, chitinase-3-like-protein 1; CDR-sob, Clinical Dementia Rating sum of boxes; MMSE, Mini-Mental State Examination; NPTX2, neuronal pentraxin 2.

For the memory factor, higher NPTX2 and C3LP1 at baseline respectively corresponded to better [R^2=.051, F=11.76, *P*<.001] or worse [R^2= .072, F=9.67, *P*=.002]

baseline performance. A NPTX2 x Time [F=8.88, *P*<.001] interaction revealed that higher baseline NPTX2 strongly corresponded to less memory decline over time relative to baseline, particularly by month 24 where NPTX2 explained 56% of the variance (**Figure 2**). By contrast, a C3LP1 main effect [F=8.851, *P*=.003], with a non-significant C3LP1 x Time interaction [F=1.73, *P*=.180], indicated that higher baseline C3LP1 showed a weak association (R^2=.04) with memory decline regardless of time. See **Supplemental Figure 1** for a trajectory curve showing predicted change in memory decline over time for NPTX2. As a confirmation analysis using 10 randomized iterations of 50% of the sample (**Supplemental Text 5**), relative effect sizes and P values for NPTX2 and C3LP1 were comparable. Exploratory interactions with covariates revealed no significant effects.

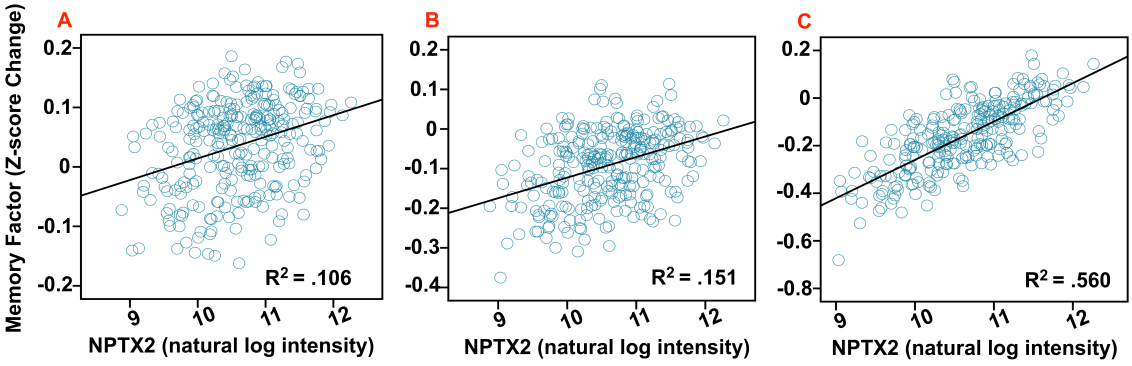


Figure 2. NPTX2 and Memory Performance across Time

Associations between baseline NPTX2 and change over time for the memory factor score relative to baseline at months 6 (A), 12 (B), and 24 (C) thereafter. Blue circles correspond to NPTX2 values. The R^2 reflects the proportion of variance in the memory factor as explained by NPTX2. Covariates included the fixed effects of age at baseline, sex, APOE ε4 genotype, and education, as well as the random effect of subject. NPTX2, neuronal pentraxin 2.

4.5. Brain: Baseline MTL volume and Atrophy over 24 Months (Mixed Models)

Next, the associations of NPTX2 and C3LP1 were investigated with baseline MTL volume and longitudinal, cumulative MTL atrophy relative to baseline with linear mixed models. Higher baseline NPTX2 [R^2=.050, F=6.91, *P*=.009] was correlated with more basal MTL volume. A NPTX2 x Time interaction [F=16.61, *P*<.001] showed that higher NPTX2 corresponded to less MTL atrophy over time, particularly by month 24 (**Figure 3A-C**). By contrast, C3LP1 showed no association with MTL volume at baseline [R^2=.008, F=0.05, *P*=.817]. A C3LP1 x Time interaction [F=12.09, *P*<.001] indicated that while baseline C3LP1 was slightly associated with atrophy over time, it was relatively modest compared to NPTX2 (**Figure 3D-F**). **Supplemental Figure 2** shows trajectory curves for predicted change in MTL atrophy over time for NPTX2 and C3LP1. These results were confirmed (**Supplemental Text 5**) when testing models with randomly selected 50% sub-samples of the cohort. Exploratory interactions with covariates revealed no significant effects.

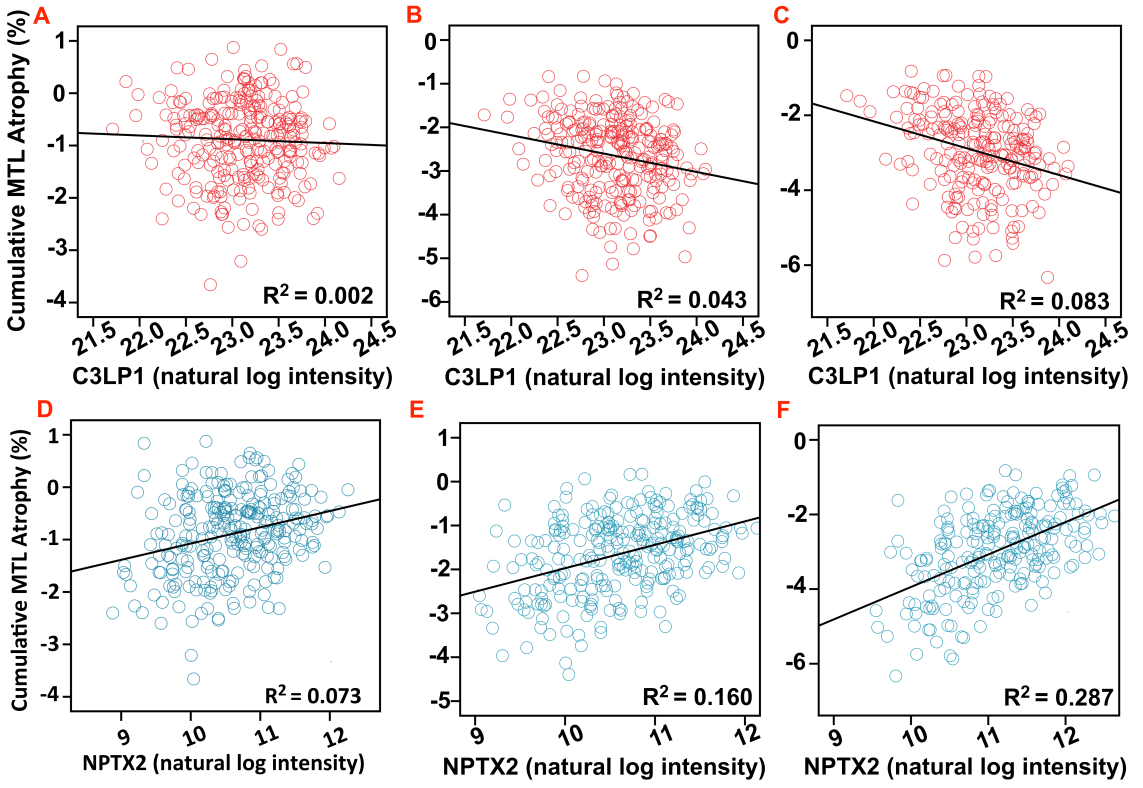


Figure 3. Mass Spectrometry Biomarkers and Medial Temporal Atrophy across Time

Associations between baseline NPTX2 (A,B,C) or C3LP1 (D,E,F) and cumulative change in medial temporal lobe (MTL) gray matter (GM) volume, expressed as a percentage relative to baseline at months 6, 12, and 24 thereafter. The blue and red circles correspond to NPTX2 and C3LP1 values respectively. The R^2 reflects the proportion of variance in MTL GM volume as explained by a given biomarker. Covariates included the fixed effects of age at baseline, sex, APOE ε4 genotype, and education, as well as the random effect of subject. C3LP1, chitinase-3-like-protein 1; NPTX2, neuronal pentraxin 2.

4.6. CSF Biomarkers: Baseline Amyloid and Tau (Mixed Models)

Finally, it was important to gauge how NPTX2 and C3LP1 were related to amyloid and tau, which are hallmarks of AD, with linear mixed models. Higher NPTX2 and C3LP1 were respectively related to a less or more AD-like CSF amyloid and tau profile. Specifically, higher NPTX2 was associated with higher CSF Aβ1-42 [β±SE=9.09±4.44 F=4.20, *P*=.041], lower total tau [β±SE=-23.89±4.07, F=34.43,

P<.001], and lower p-tau181 [β±SE=-4.34±1.45, F=8.93, *P*=.003]. By contrast, higher C3LP1 was not significantly associated with CSF Aβ1-42 [β±SE=-9.52±7.48, F=1.61, *P*=.204], but corresponded to higher total tau [β±SE=25.67±6.87, F=13.96, *P*<.001] and higher p-tau181 (β±SE= 7.71±2.46, F=9.83, *P*=.002].

Finally, we explored interactions between C3LP1 or NPTX2 and age, education, and APOE ε4 genotype, given that the covariates predicted variation in C3LP1 and NPTX2. **Supplemental Figure 3** shows that higher levels of NPTX2 were related to less amyloid pathology for non-APOE4 carriers [β±SE= 22.15±7.93, F=7.79, *P*=.006], but not for APOE4 carriers. No other interactions were significant.

CHAPTER 5: DISCUSSION & CONCLUSION

The aim of our study was to explore which established or novel pro- and anti-inflammatory CSF biomarkers from the ADNI Biomarker Core panels best predicted MTL atrophy and memory decline, as well as other AD indices affected by neuroinflammation. NPTX2 and C3LP1 consistently loaded as the only significant predictors of both MTL volume and memory performance by 24 months. They also predicted other AD aspects including global cognition and function, as well as CSF measures of amyloid and tau. Links with APOE4 status and age were found, where age has also been linked to chronic neuroinflammation over time due to age-related pro-inflammatory effects on the brain[89]. Along the AD spectrum in our study, there was a modest step-wise increase in C3LP1 and decrease in NPTX2.

These juxtaposed patterns are underscored by the global neuropsychological findings. As shown in **Figure 1** and supplemental data, higher NPTX2 reflected a significantly less AD-like pattern of global function at baseline and relative decline through month 24, while higher C3LP1 modestly corresponded to a slightly more AD-like pattern. Curiously by month 24, C3LP1 was a poor predictor for memory across time, while NPTX2 accounted for more than half of the variance among all participants. This could reflect NPTX2's role in synaptic plasticity and long-term potentiation[60,63,90]. However, NPTX2 could merely reflect the AD process, while synaptic loss reliably accompanies dementia onset[15,91,92]. Higher NPTX2 similarly correlated with less MTL atrophy over time and AD neuropathology at baseline, further highlighting its potential use to track etiopathogenesis and progression. Non-APOE4 carriers showed a relationship between Aβ and NPTX2, while APOE4 carriers did not, suggesting that the

APOE risk factor may modulate the effect of NPTX2 or an upstream mechanism. It is unclear if NPTX2 exercises a causal or correlational effect on one or more neurological and cognitive aspects of AD.

The lack of association of C3LP1 with memory scores may be due to its modest relationship with MTL atrophy over two years. Chronic neuroinflammation in AD arises from Aβ-dependent and independent activation of microglia and astrocytes[93].The release of pro-inflammatory cytokines is thought to potentiate AD pathogenesis. It is clear in this report that higher baseline C3LP1 has some modest association with AD progression, as it is significantly corresponded to global cognition, tau, and AD risk factors such as age and APOE4 status. However, baseline levels of C3LP1 were not significantly related to CSF amyloid levels. Sutphen and colleagues[79] similarly found in middle-aged, cognitively normal participants that YKL-40 (i.e., C3LP1) levels increased with age and APOE4 status, where longitudinal but not baseline associations were seen with amyloid positivity. Kester and colleagues[75] found that YKL-40 levels at baseline and longitudinally were higher in patients with MCI and AD.

Our report is particularly novel because we investigated the degree to which NPTX2 and C3LP1 track neuropathology and memory decline over time along the AD spectrum. Several limitations and strengths should be noted. Protein expression of the NPTX2 and C3LP1 peptides cannot be validated in the current dataset, as ADNI CSF samples are not readily accessible. The ADNI Biomarker Core has only assessed peptides at baseline, where longitudinal collection is needed for future work. Thus, no causal inferences can be made, and results should be considered exploratory for driving hypothesis generation. To contain type 1 error, we chose to focus structural analyses on

MTL and consequent memory performance. It could be that C3LP1 is a better predictor for global atrophy or regions other than MTL. Finally, we only analyzed subjects in ADNI, where there are to our knowledge no other readily accessible AD datasets with mass spectrometry, MRI, and neuropsychological data. For strengths, this large sample size study used an unbiased stepwise selection process and follow up stepwise validation test to select candidate biomarkers in CSF. We also highlight that NPTX2 was an excellent predictor of AD neuropathology and especially cognitive decline over time.

In conclusion, NPTX2 is a novel immunological cytokine that accounts for several neurobiological and cognitive aspects of AD, particularly cognitive decline across the AD spectrum. The microglial biomarker C3LP1, by contrast, performed modestly or did not account for AD-related indices. This research may advance the current understanding of AD etiopathogenesis, while expanding early diagnostic techniques by using novel pro-inflammatory biomarkers such as NPTX2.

CHAPTER 6: SUPPLEMENTAL TEXT

6.1. Supplemental Text 1

See the embedded table below for the full list of 21 LC/MRM-MS analytes representing 9 proteins. Each of the inflammation biomarkers is briefly described. Given that multiple peptides represent a given protein in this panel, the selection process is then described for which peptide is used in stepwise regression analyses.

Alpha-1-antitrypsin (A1AT)

A1AT, also known as alpha-1 proteinase inhibitor (A1P1), inhibits and controls serine proteases for normal biological processes[94]. Through inhibition of proteolytic enzymes, it mediates inflammatory processes and prevents unwanted tissue breakdown. A1AT operates as an anti-inflammatory protein that regulates pro-inflammatory enzymes through covalent bonding, making it a poly-functional molecule. The efficacy of A1AT can be seen in its suppression of superoxide production by activated neutrophils, which reduces oxidant-driven inflammation in tissues. A1AT also has an anti-inflammatory role through its production and release of tumor necrosis factor$-\alpha$ (TNFα). The A1AT (SVLGQLGITK) was selected because it was the only A1AT peptide to load in ADNI diagnostic prediction models[84].

CD14

A glycoprotein of the innate immune system, CD14 is found on the surface of many Toll-Like Receptor 4 (TLR4) expressing cells, such as macrophages and neutrophils. It acts like a receptor for lipopolysaccharide (LPS) complexes and LPS-

binding protein. It is involved in LPS-induced cytokine activation. It is also important for TNFAα expression[95]. CD14 is a functional protein that exhibits its effects through signal transmission for cytokines[96]. CD14 (SWLAELQQWLKPGLK) was selected because it was the only CD14 peptide to load in ADNI diagnostic prediction models[84].

Complement 3 (CO3)

CO3 is a functional serum protein that plays a central role in the activation of the classic, alternative, and lectin pathways of the complement system, and promotes inflammation-induced immune reaction[97]. It beneficially influences innate immunity by promoting phagocytosis, supporting inflammatory responses, and instructing the adaptive immune response to select appropriate antigens for a humoral response. Uncontrolled activation of this peptide can also have negative effects. CO3 (IHWESASLLR) was selected because it was the only peptide to load for predicting MCI conversion[84], and it best accounted for variance in memory decline and MTL atrophy by 24 months. While many other peptides were available for other complement proteins, CO3 is of central importance to regulating the complement system.

Chitinase 3-like Protein 1 (C3LP1)/YKL-40

C3LP1 is a pro-inflammatory cytokine found in inflammatory environments that is secreted by chondrocytes, differentiated macrophages, neutrophils, and synovial cells. It works to stimulate an inflammatory response from immune system cells and connective tissue cells. C3LP1's pro-inflammatory effects are sequential to its inhibition of the vital processes of immune cell apoptosis[78]. Levels of this biomarker are found to be increased

in chronic inflammatory disorders[78]. Elevated levels of C3LP1 in CSF have recently been associated with chronic neuroinflammation and resultant conditions[98]. During neuroinflammatory processes, C3LP1 is found to be induced in astrocytes by the inflammatory cytokines IL-1β and TNFα[98]. Inflammatory cytokines TNF and interleukin-1 can induce steady state levels of C3LP1[99]. C3LP1 (ILGQQVPYATK) was selected because preliminary stepwise regression analyses suggested that it explained the most variance in memory decline and MTL atrophy by 24 months.

Interleukin-18 (IL-18)

IL-18 is a pro-inflammatory cytokine that establishes innate cell-mediated immunity and an inflammatory response in the body through T-cell activation, induction of Interferon γ (IFNγ), granulocyte macrophage (GM-CSF), TNF and IL-1 cells and up-regulation of chemokine receptors[100]. It has been implicated to have increased levels in AD brains(Sutinen EM, 2012). IL-18 was found to increase amyloid-beta production, a hallmark of AD[101]. IL-18 acts bilaterally as a pro-inflammatory cytokine and as a strong inducer of atopic immune responses. IL-18 (LWEGSTSR) was the only available peptide for analysis.

Osteopontin (OSTP)

OSTP, a highly acidic secreted phosphoprotein, is found in bone and tissues. It is a regulator of immune system signaling and inflammatory responses through chemotactic cell recruitment to inflammatory sites. OSTP is expressed by various immune cells and is found to be upregulated in many immune system responses[102]. Immune modulation and

OSTP antibody production occur when OSTP acts as a cytokine through interaction with cellular and humoral receptors of the immune system. This protein is a participant in the pathogenesis of many autoimmune related diseases. OSTP (AIPVAQDLNAPSDWDSR) was the only available peptide for analysis.

C-Reactive Protein (CRP)

CRP is a pentameric serum protein made in response to IL-6 and whose levels markedly rise in response to systemic inflammation. CRP's inflammatory response in chronic disease states is instigated by the release of pro-inflammatory cytokines. The primary role of CRP is to regulate acute inflammation by altering the equilibrium between pro- and anti-inflammatory cytokines and activating complement proteins[103]. CRP (ESDTSYVSLK) was the only available peptide for analysis.

Neuronal Pentraxin 1 (NPTX1)

NPTX1 is a protein of the neuronal long pentraxin family that is similar to the small pentraxins, such as CRP. It is homologous to NPTX2. It is produced by neurons in response to low activity, affecting mitochondrial function and contributing to neurodegeneration through apoptosis[104]. NPTX1 also plays a role in excitatory synaptic plasticity, where amyloid-beta treatment increases NPTX1 and leads to synapse loss[105]. NPTX1 has been labeled as a dual action protein as seen in it's both beneficial synapse formation and negative neurodegeneration effects[106]. NPTX1 (LENLEQYSR) was the only peptide to load in ADNI diagnostic prediction model[84].

Neuronal Pentraxin 2 (NPTX2)

NPTX2, another secreted neuronal long pentraxin, functions primarily to facilitate excitatory synaptogenesis by facilitating aggregation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. It is involved in formation of synapses, synaptic plasticity and plays a role in eradicating synaptic debris[106]. NPTX2 (TESTNALLQR) was selected for analysis based on its predictive value of distinguishing MCI from AD patients[84].

Protein	Peptide
A1AT	AVLTIDEK
A1AT	LSITGTYDLK
A1AT	SVLGQLGITK
CD14	AFPALTSLDLSDNPGLGER
CD14	FPAIQNLALR
CD14	SWLAELQQWLKPGLK
CO3	IHWESASLLR
CO3	LSINTHPSQKPLSITVR
CO3	TELRPGETLNVNFLLR
CO3	TGLQEVEVK
CO3	VPVAVQGEDTVQSLTQGDGVAK
C3LP1	ILGQQVPYATK
C3LP1	SFTLASSETGVGAPISGPGIPGR
C3LP1	VTIDSSYDIAK
IL18	LWEGSTSR
OSTP	AIPVAQDLNAPSDWDSR
CRP	ESDTSYVSLK
NPTX1	FQLTFPLR
NPTX1	LENLEQYSR
NPTX2	LESLEHQLR
NPTX2	TESTLNALLQR

6.2. Supplemental Text 2

Biomarker selection using randomized 50% sub-samples (stepwise regression and lasso regression)

These supplemental analyses were done to further validate the stepwise selection of NPTX2 and C3LP1 as the only significant predictor variables using the full n=285 sample. Validation occurred through 10 randomized iterations, where we split the sample by 50% to first conduct biomarker selection and model creation, followed by model validation using the other 50% of the sample. Specifically, for each randomized iteration, we put the 9 pro-inflammatory CSF mass spectrometry biomarkers into a stepwise block after entering covariates in the first block. Using inclusion and exclusion criteria of Alpha < .05 and Alpha > .10, we determined which biomarkers significantly loaded onto the model. For models where NPTX2 and/or C3LP1 loaded, we then conducted repeated measures linear mixed models, to see to what degree NPTX2 and/or C3LP1 predicted our outcomes of interest (memory decline; brain atrophy) at Months 0, 6, 12, and 24 (see **Supplemental Text 5**).

The table below illustrates biomarker selection results for the 10 randomized iterations.

Supplemental Text 2 Embedded Table. Biomarker Selection of 10 Random Iterations

Memory				Atrophy				
Iteration	1st biomarker	2nd biomarker	3rd biomarker	Iteration	1st biomarker	2nd biomarker	3rd biomarker	4th biomarker
1	NPTX2	C3LP1	IL-18	1	NPTX2	C3LP1		
2	NPTX2	C3LP1		2	NPTX2	C3LP1		
3	NPTX2	C3LP1		3	C3LP1	CD-14		
4	NPTX2	Osteopontin		4	NPTX2	C3LP1		
5	None	None		5	NPTX2	C3LP1	A1antitropsin	
6	NPTX2	C3LP1		6	NPTX2	C3LP1		
7	NPTX2	C3LP1		7	CRP			
8	NPTX1	C3LP1	IL-18	8	NPTX2	C3LP1		
9	NPTX2	C3LP1		9	NPTX2	C3LP1		
10	NPTX2			10	NPTX2	C3LP1	CD-14	Osteopontin

The table indicates that for predicting memory decline at month 24, stepwise regression selected C3LP1 7 out of 10 random iterations and NPTX2 8 out of 10 random iterations.

For temporal atrophy at month 24, stepwise regression selected C3LP1 9 out of 10 random iterations and NPTX2 8 out of 10 random iterations. When selected, NPTX2 and C3LP1 loaded as the first and second selected biomarkers respectively. These results suggest that NPTX2 and C3LP1 consistently loaded onto the model, validating their selection using the full cohort.

In addition to our 50%/50% model selection and validation, we also performed Lasso regression for both temporal atrophy and memory decline. Lasso regression confirmed that NPTX2 and C3LP1 were consistently selected as the only significant predictors in the first six most stringent statistical models tested (tables not shown). These Lasso analyses further validated our initial stepwise regression process using the full sample and our supplementary stepwise biomarker selection analyses using 50% of the sample.

6.3. Supplemental Text 3

In these supplementary analyses, we compared stepwise regression performance of inflammation-related biomarkers from two ADNI Biomarker Consortium CSF Proteomics Project panels. We specifically examined if the selected LC/MRM-MS NPTX2 (TESTLNALLQR) and C3LP1 (ILGQQVPYATK) peptides performed better than or comparable to inflammation-associated proteins in CSF from a Rules Based Medicine (MyriadRBM) multiplex assay. The outcomes of interest were MTL atrophy and memory decline by month 24 relative to baseline. Covariates were put in using the ‘enter’ method in the first model step and included age, sex, education, clinical diagnosis

at baseline, and APOE Status. Predictors were then put in using the 'stepwise' method in the second model step.

Methods for the MyriadRBM panel are currently described in the Consortium's white paper (See "2011Dec19 Biomarkers Consortium Data Primer PDF-2" under 'Biospecimen Results' at http://ida.loni.usc.edu). Briefly, the Luminex xMAP immunoassay panel ("discovery MAP") was run on 159 CSF analytes relevant to Alzheimer's etiology or progression. Extensive quality control (QC) was performed including test/retest samples, assessing volumetric and mechanical functionality of the system, and confidence levels of analyte results in comparison to the MyriadRBM protocol. We obtained values from the ADNI CSF QC Multiplex data set. For exploration purposes, we examined all 27 inflammation-related analytes (see **Table e-1**) regardless of QC confidence intervals. Many QC analytes had no sample in the detectable range.

For MTL atrophy by 24 months, among 229 participants with longitudinal brain data, MyriadRBM biomarkers were first assessed. Interleukin-3 significantly loaded onto the model [F Change(1,222) = 9.179, p = .003]. Adjusted R-Squares indicated that interleukin-3 explained an additional 1.8% of the variance beyond the 48.5% attributed to covariates. In a subsequent stepwise model, among 227 participants with both MyriadRBM and MRM-MS data, interleukin-3 was added in the same step with NPTX2 and C3LP1. Both NPTX2 [F Change(1,220) = 17.623, p < .001] and C3LP1 [F Change(1,220) = 16.786, p < .001] loaded significantly, whereas interleukin-3 was a marginal contributor and therefore excluded [t = 1.921, p = .056].

For memory decline by 24 months, among 260 participants with longitudinal neuropsychological data, MyriadRBM biomarkers were first assessed. Angiopoietin-2 [F Change (1,253) = 7.873, p = .005] and RANTES [F Change (1,252) = 4.576, p = .033] both loaded as significant predictors. These analytes explained an additional 1.2% and 1.1% of the variance in memory decline beyond 16.3% attributed to covariates. In a subsequent stepwise model, among 258 participants with both MyriadRBM and MRM-MS data, Angiopoietin-2 and RANTES were added in the same step with NPTX2 and C3LP1. All of the predictors loaded significantly [NPTX2: F Change (1,251) = 12.918, p < .001; C3LP1: F Change (1,250) = 5.173, p = .024; Angiopoietin-2: F Change (1,249) = 5.086, p = .025; RANTES: F Change (1, 248) = 4.357, p = .038], respectively explaining an additional 3.8%, 1.3%, 1.3%, and 1.0% of the variance beyond the 16.4% attributed to covariates.

These results suggest that C3LP1 and NPTX2 are consistently implicated in both MTL atrophy and memory decline in ADNI. Therefore, we continued to use these peptide biomarkers in subsequent analyses. By contrast, MyriadRBM biomarkers appear to be useful predictors only for memory decline. Several limitations should be acknowledged. The MyriadRBM panel surveyed many inflammatory cytokines, chemokines, tumor necrosis factors, and interferons. Unfortunately, detection limits precluded analysis of many potentially viable biomarkers in CSF. We also did not comparatively test panel differences for other brain regions that exhibit Alzheimer's-related atrophy over time, such as prefrontal cortex.

6.4. Supplemental Text 4

Across time, NPTX2 x Time interactions for MMSE [F=6.787, *P*<.001], CDR-sob [F=10.497, *P*<.001], and ADAScog-11 [F=9.019, *P*<.001] indicated that higher levels predicted less global decline. For example, by month 24, higher baseline NPTX2 predicted higher MMSE [β±SE=1.49±0.33, F=6.79, *P*<.001], lower CDR-sob [β±SE=-0.98±0.19, F=10.50, *P*<.001], and lower ADAScog-11 scores [β±SE=-2.65±0.54, F=9.02, *P*<.001]. The converse pattern was seen with the C3LP1 x Time interactions for MMSE [F=3.911, *P*=.021], CDR-sob [F=6.439, *P*=.002], and ADAScog-11 [F=7.239, *P*=.001]. For example, by month 24, higher C3LP1 levels predicted lower MMSE [β±SE=-1.85±0.53, F=3.91, *P*=.021], higher CDR-sob [β±SE=0.80±0.30, F=6.44, *P*=.002], and higher ADAScog-11 [β±SE=2.38±0.86, F=7.24, *P*<.001]. Exploratory interactions with covariates revealed no significant effects after Holm-Bonferroni correction.

6.5. Supplemental Text 5

Confirmatory tests of biomarkers using randomized 50% sub-samples (mixed models)

These supplemental analyses were done to confirm models created from the biomarker selection step (see **Supplemental Text 2**). The repeated measures linear mixed models analyses for the table below were performed using a randomly selected 50% of subjects from the n=285 cohort per iteration. The term of interest was a Predictor x Time interaction, to determine to what degree a predictor explained variance in the outcome over time. The number of confirmation test iterations for NPTX2 and C3LP1 was directly proportional to the number of times NPTX2 and/or C3LP1 were selected in the model generation step using the other randomly selected 50% of subjects from the full cohort.

For example, as shown in **Supplemental Text 2**, NPTX2 and C3LP1 were chosen in the model generation phase 8 out of 10 and 7 out of 10 times respectively. Therefore, we conducted 8 randomized confirmation tests to regress NPTX2 against memory decline over time, and similarly conducted 7 randomized confirmation tests to regress C3LP1 against memory decline over time.

The table below shows the R-squared value and p-value per random iteration for NPTX2 and C3LP1. We also list the mean R-squared value and p-value. For simplicity, we only report the R-squared in predicting medial temporal atrophy and memory decline between months 12 to 24. P values for the Predictor by Time interaction are noted below each R-squared for a given random iteration.

Supplemental Text 5 Embedded Table. R^2 value and p-value per random iteration for NPTX2 and C3LP1.

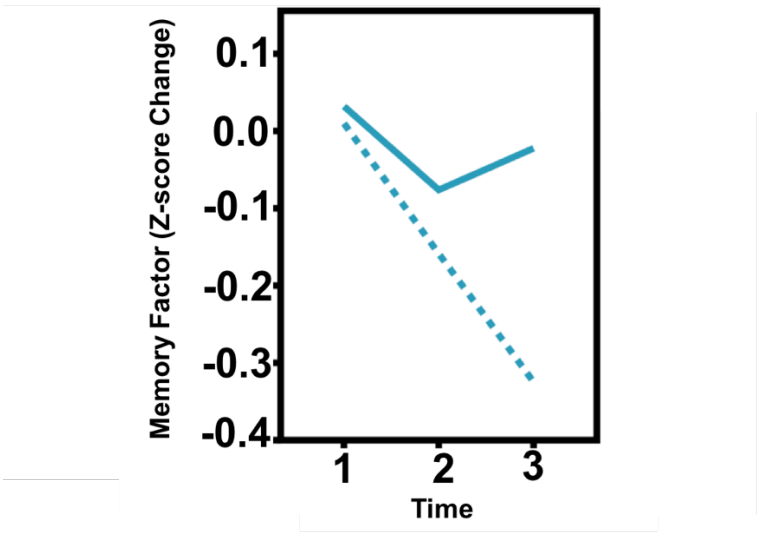
Memory Decline

NPTX2	**Iteration 1**	**Iteration 2**	**Iteration 3**	**Iteration 4**	**Iteration 5**	**Iteration 6**	**Iteration 7**	**Iteration 8**	**Mean**
M12 to M24 R-squared value	0.34	0.543	0.407	0.502	0.293	0.266	0.218	0.501	0.384
NPTX2 * Time: p value	0.003	< .001	< .001	< .001	0.003	0.007	0.003	< .001	< .001
C3LP1									
M12 to M24 R-squared value	0.04	0.065	0.082	0.041	0.03	0.037	0.023		0.045
C3LP1 * Time: p value	0.312	0.136	0.034	0.267	0.424	0.232	0.608		0.288

Medial Temporal Atrophy

NPTX2	**Iteration 1**	**Iteration 2**	**Iteration 3**	**Iteration 4**	**Iteration 5**	**Iteration 6**	**Iteration 7**	**Iteration 8**	**Iteration 9**	**Mean**
M12 to M24 R-squared value	0.125	0.251	0.434	0.214	0.243	0.123	0.28	0.317		0.248
NPTX2 * Time: p value	0.003	0.007	0	0	0	0	0.003	0.003		< .001
C3LP1										
M12 to M24 R-squared value	0.159	0.123	0.122	0.054	0.049	0.157	0.132	0.034	0.058	0.099
C3LP1 * Time: p value	< .001	0.015	< .001	0.002	< .001	< .001	< .001	0.001	0.024	0.005

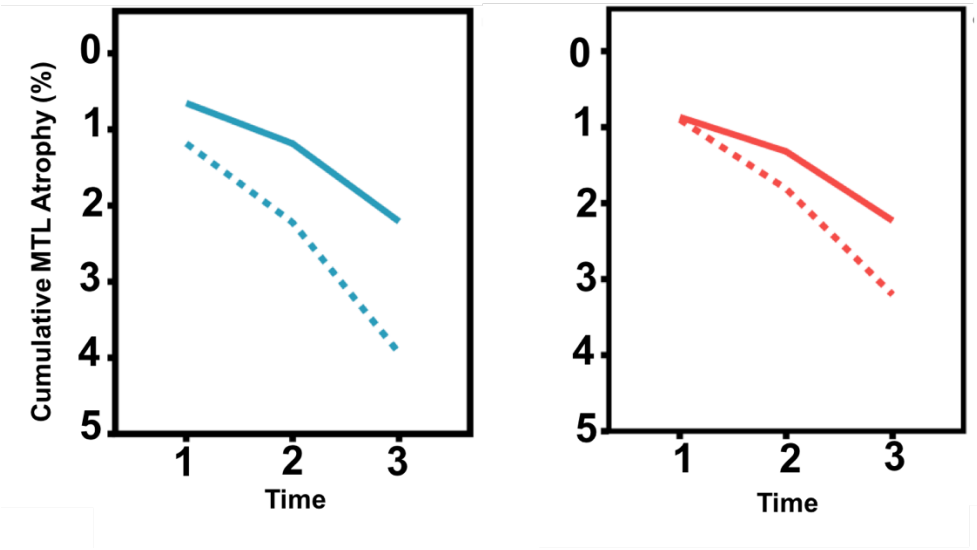
6.6. Supplemental Figure 1.



Supplemental Figure 1. NPTX2 and Memory Performance across Time

Associations between baseline NPTX2 and change over time for the memory factor score relative to baseline at months 6 (Time 1), 12 (Time 2), and 24 (Time 3). The solid and dotted blue lines indicate mean atrophy over time for subjects at 1-2SD above or below the mean for NPTX2 respectively. Please note that C3LP1 was not a significant predictor of change in the memory factor over time. NPTX2, neuronal pentraxin 2.

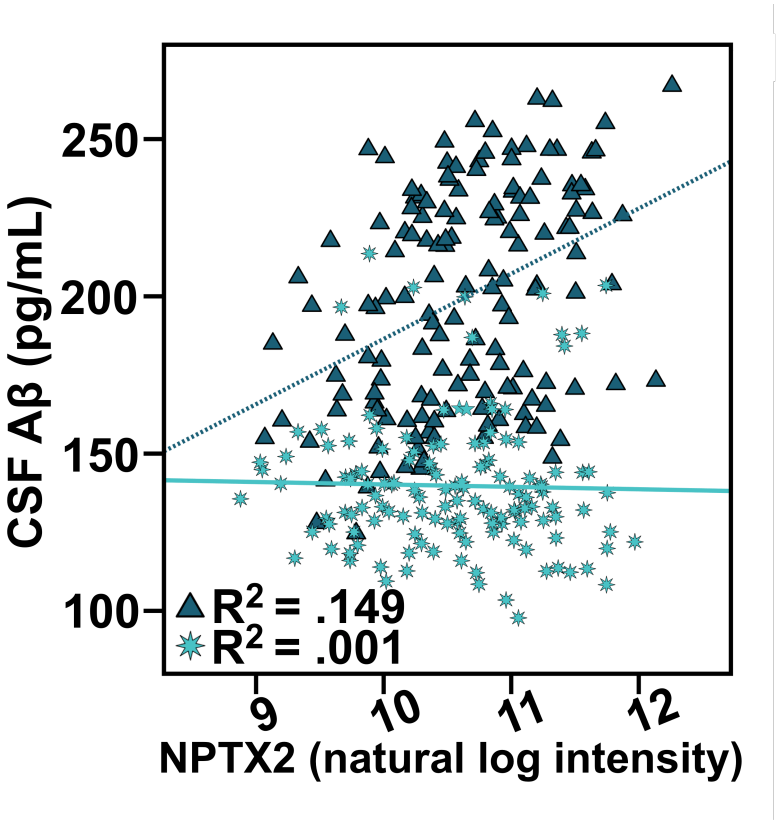
6.7. Supplementary Figure 2.



Supplemental Figure 2. Mass Spectrometry Biomarkers and Medial Temporal Atrophy across Time

Associations between baseline NPTX2 (left graph) or C3LP1 (right graph) and change in medial temporal lobe (MTL) gray matter volume, expressed as a percentage relative to baseline and months 6 (Time 1), 12 (Time 2), and 24 (Time 3). The solid and dotted blue lines indicate mean atrophy over time for subjects at 1-2SD above or below the mean for NPTX2 respectively. The solid and dotted red lines indicate mean atrophy over time for subjects at 1-2SD above or below the mean for C3LP1 respectively. C3LP1, chitinase-3-like-protein 1; NPTX2, neuronal pentraxin 2.

6.8. Supplemental Figure 3



Supplemental Figure 3. Modulation of NPTX2 and CSF Amyloid Associations by APOE Genotype

The association between baseline CSF Aβ 1-42 and NPTX2t among non-APOE4 or APOE4 carriers. The star and triangle shapes indicate values for non-APOE4 and APOE4 carriers respectively. The R^2 reflects the proportion of variance in amyloid burden as explained by the associations of NPTX2 for APOE4 and non-APOE4 carriers. Covariates included age at baseline, sex, education, APOE ε4 genotype, and baseline clinical diagnosis. NPTX2, neuronal pentraxin 2.

6.9. Supplemental Table 1

Supplemental Table 1. Inflammation-Related Biomarkers

Supplemental Table 1. Inflammation-Related Biomarkers

Rules Based Medicine (RBM) Biomarker	Unit
Alpha-1-Antitrypsin	mg/mL
Angiopoietin-2	ng/mL
C-Reactive Protein	ug/mL
CD 40 antigen	ng/mL
Chemokine CC4	ng/mL
Complement C3	mg/mL
Fas Ligand	pg/mL
Immunoglobulin A	mg/mL
Interferon gamma Induced Protein 10	pg/mL
Interleukin-16	pg/mL
Interleukin-25	pg/mL
Interleukin-3	ng/mL
Interleukin-6 receptor	ng/mL
Interleukin-8	pg/mL
Macrophage Colony-Stimulating Factor 1	ng/mL
Macrophage Inflammatory Protein-1 beta	pg/mL
Macrophage Migration Inhibitory Factor	ng/mL
Monocyte Chemotactic Protein 1	pg/mL
Monocyte Chemotactic Protein 2	pg/mL
Monokine Induced by Gamma Interferon	pg/mL
Neutrophil Gelatinase-Associated Lipocalin	ng/mL
Osteopontin	ng/mL
Resistin	ng/mL
T Lymphocyte-Secreted Protein I-309	pg/mL
T-Cell-Specific Protein RANTES	ng/mL
TNF-Related Apoptosis-Inducing Ligand	ng/mL
TNF Receptor 2	ng/mL

TNF= Tumor Necrosis Factor

CHAPTER 7: REFERENCES

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